Quantitative Analysis of Oxyresveratrol in Different Plant Parts of *Morus* Species and Related Genera by HPTLC and HPLC

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Key Words

HPLC
HPTLC
Oxyresveratrol
***Morus* species**

Summary

Quantitative analysis of oxyresveratrol in different parts of *Morus* species by HPTLC and HPLC is described. The methods were validated for selectivity, extraction efficiency, sensitivity, accuracy, and intra-day and inter-day reproducibility. Extraction efficiency was in the range 100 ± 3.2%. Limits of detection and quantification for oxyresveratrol in plant samples were 50 and 200 ng per band, respectively, by HPTLC and 0.3 and 1.0 μg mL^{-1}, respectively, by HPLC. The amount of oxyresveratrol was higher in stems than in leaves. It was not detected in leaves of *Morus rubra* L, and was detected only in roots and bark of *Morus alba* L. These methods, which were found to be simple and sensitive with good precision and reproducibility, were also used for analysis of oxyresveratrol in other related species and genera.

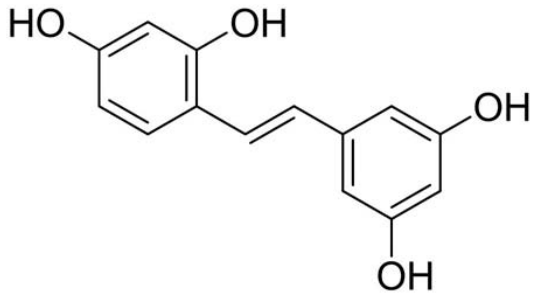

Figure 1

The structure of oxyresveratrol.

1 Introduction

Oxyresveratrol (*trans*-2,3′,4,5′-tetrahydroxystilbene, **Figure 1**), a naturally occurring compound found in *Morus* species, has received much attention because of its interesting bioactivity. *Morus rubra* L., which belongs to the Moraceae family and is commonly known as the red mulberry, is a species native to eastern North America, from northernmost Ontario and Vermont south to southern Florida and west to South Dakota and central Texas. Although common in the United States, it is listed as an endangered species in Canada [1, 2]. As part of our program to search for chemical and/or biological marker compounds for dietary supplements, oxyresveratrol was isolated from the stems of *M. rubra* L. and identified. Recent investigations have revealed that the fruit and leaves of mulberry plants contain many bioactive compounds, for example alkaloids, polyphenols, anthocyanins, and flavonoids [3–5], which have been reported to be beneficial for various health conditions. Oxyresveratrol, which is found in many plant species including grapes, peanuts, and mulberries [6], has an inhibitory effect on tyrosinase, limiting melanin biosynthesis, and is used as a cosmetic material and as a medical agent for hyperpigmentation disorders [7, 8]. Other biological activity associated with oxyresveratrol include antiherpetic, anti-HIV, anti-inflammatory, and anti-oxidant, and it is recommended for treatment of neurodegenerative disorders [9–15]. This biological activity is indicative of several areas of therapeutic potential for oxyresveratrol, and the presence of the compound in the *Morus* species suggests they may be an important natural source.

Various methods for quantification of oxyresveratrol have been described, but quantitative analysis of *M. rubra* L. has not been reported. Previously reported analytical methods for oxyresveratrol include gravimetric [16] and high-performance liquid chromatographic (HPLC) [5, 17, 18] techniques. In this manuscript we describe simple, rapid, and precise HPTLC and HPLC methods for quantitative analysis of oxyresveratrol in various parts of the plant *M. rubra* L. A chemical fingerprint generated by HPTLC and HPLC–UV for *M. rubra* L. was compared with those of various other species and plants in related genera.

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2 Experimental

2.1 Plant Materials and Solutions

The ground stems of *M. rubra* L. (MR-1) (G. Walters 820), bark of *M. alba* L. (MA-3) (J. Stone 4247), stems of *M. alba* L. (MA-6), (J. Stone 4178), fruits of *M. alba* L. (MA-7) (J. Stone 4178), stems of *M. rubra* L. (MR-1) (G. Walters 820), leaves–stems of *M. rubra* L. (MR-2) (G. Walters 817), and leaves of *M. rubra* L. (MR-3) (G. Walters 820) were obtained from Missouri Botanical Garden, Missouri, USA. The bark of *M. alba* L. (MA-1) (CON200700-1-C), roots of *M. alba* L. (MA-2) (CON200700-1-A), xylem of *M. alba* L. (MA-4) (CON200700-1-B), leaves of *M. alba* L. (MA-5) (CON200700-1-D), leaves of *Maclura pomifera* (MP-1) (CON1007-1-A); fruits of *M. pomifera* (MP-2) (CON100700-1-C), and roots of *M. pomifera* (MP-3) (CON100700-1-B) were collected by Bryan Connolly and voucher specimens are deposited at the Pullen Herbarium, The University of Mississippi. Leaf–twigs of *Trymatococcus amazonicus* (TA-1) (IBE11924-C) and stems of *Ficus maxima* (FM-1) (IBE11571) were collected by Sydney McDaniel, and voucher specimens are deposited at Sessums Mississippi, USA. Sample specimens of all materials are deposited at the National Center for Natural Products Research (NCNPR), University of Mississippi, Mississippi, USA.

All chemicals and solvents were HPLC-grade.

All solutions were freshly prepared. A stock solution of oxyresveratrol standard was prepared at a concentration of 1.0 mg mL^{-1} in methanol. This stock solution was further diluted with methanol to furnish a standard solution of 1 μg mL^{-1}.

2.2 Sample Preparation

Dry plant samples (0.5 g) were sonicated in 2.5 mL methanol for 20 min (Fisher Scientific, USA, sonicator) followed by centrifugation for 10 min at 3300 rpm. The supernatant was transferred to a 25-mL volumetric flask. The procedure was repeated four times and the extracts were combined. The final volume was adjusted to 25 mL with methanol and mixed thoroughly. Before analysis, an adequate volume (ca. 2 mL) was passed through a 0.2-μm nylon membrane filter, by use of a Millex (Millipore, USA) syringe-driven filter unit.

2.3 HPTLC

2.3.1 Procedure

HPTLC was performed on 20 cm × 10 cm and 10 cm × 10 cm plates coated with 200 μm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany). Before use the plates were prewashed with methanol and dried for 5 min at 120°C. Standard and sample solutions were applied to the plates as 8 mm bands by means of a CAMAG (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100-μL syringe and connected to a Nitrogen tank. The track spacing was 11.3 mm, the distance from the left edge 13 mm, and the distance from the bottom edge 10 mm. The delivery speed was 2 μL s^{-1} and the application volume 4–10 μL. Other settings were left at the default values. Each plate accommodated 17 tracks of samples from *Morus* species and related genera, and oxyresveratrol standard.

Plates were developed with hexane–ethyl acetate–chloroform–methanol 3.0:2.0:3.4:1.6 (*v/v*) as mobile phase in an Analtech (USA) 20 cm × 10 cm twin-trough glass chamber lined with Whatman filter paper (20 cm × 10 cm) and previously saturated with mobile phase vapor for 20 min. The development distance was 85 mm. Development was at ambient temperature (23°C) and 60–65% relative humidity (measured by use of an Acurite instrument).

After inspection of the plates under UV and visible light, quantitative evaluation was by scanning densitometry at 327 nm with a CAMAG TLC scanner. The slit dimensions were 6.0 mm × 0.4 mm, the scanning speed 20 nm s^{-1}, and data resolution 100 mm $step^{-1}$. A CAMAG Reprostar 3 with DigiStore 2 Digital Documentation System and winCATS 4 software ver. 1.4.3 was used for imaging and archiving the chromatograms.

2.3.2 Validation

The UV spectrum of oxyresveratrol was obtained from a developed HPTLC plate and 327 nm was selected as detection wavelength. The presence of oxyresveratrol in different plant samples was confirmed by comparing R_F values and absorption spectra with those of oxyresveratrol. To assess specificity the peak purity of oxyresveratrol was determined by correlating the spectrum for each plant species scanned at the peak start, peak apex, and peak end positions of the band.

Accuracy was assessed by measurement of recovery. Samples MA-1, MA-2, MR-3, MR-1, and MR-2 were spiked with 10 and 20 μg mL^{-1} of the standard solution and then extracted and analyzed under the optimized conditions.

Intra-day and inter-day precision of the method was determined by extracting and analyzing five samples (MA-1, MA-2, MR-3, MR-1, and MR-2) on three consecutive days under optimized conditions.

To assess stability, sample extract solutions were prepared, stored at room temperature for up to 24 h, then analyzed on the same HPTLC plate. After development the chromatogram was evaluated for additional bands. Band stability was assessed by inspecting the developed plates after 30 min and 1, 2, 4, and 12 h.

A calibration plot of peak area against concentration was prepared after chromatography of five different concentrations in the range 200–1000 ng per band.

Limits of detection (LOD) and quantification (LOQ) were determined by serial dilution and were based the amounts for which the signal to noise ratios of 3:1 and 10:1, respectively.

2.4 HPLC

HPLC was performed with a Waters (Milford, MA, USA) 2695 Alliance Separations Module. Compounds were separated on a 150 mm × 4.6 mm, 4 μm particle size, Synergi Fusion-RP 80 Å column from Phenomenex (Torrance, CA, USA). The mobile phase was a gradient prepared from acetonitrile (component A) and water (component B), both containing 0.1% acetic acid. The gradient program was: 0–15 min, 5% A:95% B to 47% A:53% B; 15–20 min, 47% A:53% B to 100% A. Re-equilibration with 5% A:95% B then followed for 15 min. The flow rate was 1.0 mL min^{-1}, the injection volume 10 μL, and the column temperature 25°C. Peaks were assigned by spiking samples with compound standards and comparison of UV spectra and retention times.

A calibration plot of peak area against concentration was prepared after chromatography of five different concentrations in the range 1–200 μg mL^{-1}. LOD and LOQ were determined as described above.

3 Results and Discussion

3.1 Optimized Mobile Phases for HPTLC and HPLC

The mobile phase hexane–ethyl acetate–chloroform/methanol 3.0:2.0:3.4:1.6 (*v*/*v*) selected for HPTLC analysis resulted on good resolution of oxyresveratrol at R_F 0.31 ± 0.01.

Optimum HPLC conditions were obtained after running different mobile phases with a reversed phase C_{18} column. The best results were obtained by use of a Synergi Fusion-RP 80 Å column with a mobile phase gradient prepared from water and acetonitrile, both containing 0.1% acetic acid. Variation of the column temperature between 25 and 40°C did not cause significant change in the resolution, although changes in retention time were observed. The column was used at 25°C and flow rate of 1.0 mL min^{-1}.

3.2 Method Validation

The methods were validated for accuracy, precision, linearity, and limit of detection in accordance with USP guidelines, an important for assessment of method quality.

In TLC, densitometry at 327 nm revealed there was no overlap of oxyresveratrol with any other components of the analyzed samples. The method was validated for specificity, linearity, precision (repeatability), recovery, and accuracy. Non-linear regression was used for curve fitting. The polynomial regression equation for the calibration range 200–1000 ng per band, on the basis of peak area, was $Y = 905.706 + 19.532X + -0.007X^2$ (regression coefficient (r) = 0.9995). In HPLC the five point calibration curve for oxyresveratrol was indicative of a linear correlation between concentration and peak area in the range 1–200 μg mL^{-1}. The linear regression equation was $Y = 3.45 \times 10^4 - 6.04 \times 10^4$ ($r^2 > 0.999$).

In HPTLC the LOD and LOQ were 50 and 200 ng per band, respectively. The recovery of the method was in the range 97–103.2%, confirming both the accuracy of the method and the integrity of the extraction procedure. In HPLC the LOD and LOQ were 0.3 and 1.0 μg mL^{-1}, respectively.

All standards and samples were analyzed in triplicate. Results were highly reproducible with low standard error. Intra-day and inter-day variation of the assays were determined on three consecutive days with three replicates each resulted in RSD values consistently below 5.0%, with maximum RSD of 4.13 and 3.23% by HPTLC and HPLC, respectively.

Within day CVs for the replicate analyses (n = 3) were in the range 1.08–4.13% for HPTLC and 2.12% for HPLC. CVs for day-to-day replicates (n = 9) were between 2.08 and 2.81% for HPTLC and 2.22% for HPLC. Peak purity and identity were verified by studying UV spectra, and by spiking samples with the reference compound. No indications of impurities were found.

Table 1

Amounts (%, *w*/*w*) of oxyresveratrol in different plant samples by use of HPTLC and HPLC.

Plant Name	Plant Part	HPTLC[a)]	HPLC[a)]
Morus alba L.	Roots	0.30 ± 0.009	0.27 ± 0.0054
Morus alba L.	Bark (BK)	0.27 ± 0.01	0.26 ± 0.0007
Morus alba L.	Leaves	ND	ND
Morus alba L.	Xylem	ND	ND
Morus alba L.	Stems	ND	ND
Morus alba L.	Fruits	ND	ND
Morus alba L.	Bark	0.10 ± 0.002	0.08 ± 0.0024
Morus rubra L.	Stems	0.62 ± 0.01	0.60 ± 0.0001
Morus rubra L.	Stems–leaves	0.31 ± 0.011	0.29 ± 0.0101
Morus rubra L.	Leaves	ND	ND
Maclura pomifera	Leaves	ND	ND
Maclura pomifera	Fruits	ND	ND
Maclura pomifera	Roots	ND	DUL
Trymatococcus amazonicus	Leaves–twigs	ND	ND
Ficus maxima	Stems	ND	ND

ND, not detected; DUL, detected, below the LOQ; BK, outer layer of bark

[a)]Mean ± standard deviation (n = 3)

There was no indication of compound instability in sample solutions. Sample extracts were stable on HPTLC plates for at least one day. Two-dimensional chromatography using the same mobile phase system was used to discover decomposition occurring during sample application and development. If decomposition occurs during development, peak(s) of the decomposition product(s) should be obtained in both the first and second directions of the run. No decomposition was observed during sample application and development.

3.3 Analysis of Plant Samples

The methods were applied for quantification of oxyresveratrol in various *Morus* species and related genera; the results obtained are listed in **Table 1**. The oxyresveratrol content of the stems, bark, and roots of *M. rubra* L and *M. alba* L. were 0.08–0.60%. Oxyresveratrol was not identified in leaves, fruits, or xylem of *M. alba* L. or the leaves of *M. rubra* L. *Song et al.* [5] recently reported the presence of oxyresveratrol in the leaves of *M. alba* L. The amounts detected were not significant and variations occurred among the samples collected and depended on the stage of the harvest. In comparison with our analytical data, the bark and stem were found to contain more oxyresveratrol than found in the leaves and fruits. With the LC–UV method, identification of oxyresveratrol in *Morus* and related genera was based on retention times, comparison of UV spectra, and by spiking the extracts with reference compounds. Chemical fingerprint analysis by HPTLC and HPLC resulted in distinct profiles for the different *Morus* species and related genera

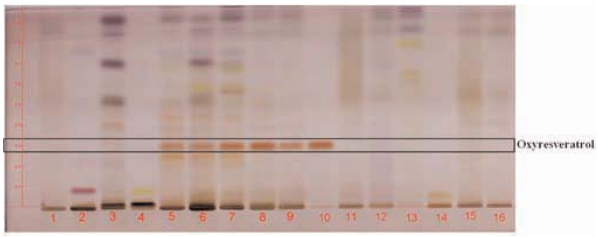

Figure 2

HPTLC comparison of *Morus* species with other related genera under visible light. Tracks: 1, leaves of *M. alba* L.; 2, xylem of *M. alba* L.; 3, stems of *M. alba* L.; 4, fruits of *M. alba* L.; 5, roots of *M. alba* L.; 6, bark (BK) *M. alba* L.; 7, bark of *M. alba* L.; 8, stems of *M. rubra* L.; 9, stems–leaves of *M. rubra* L.; 10, oxyresveratrol; 11, leaves of *M. rubra* L.; 12, leaves of *Maclura pomifera*; 13, fruits of *M. pomifera*; 14, roots of *M. pomifera*; 15, leaves–twigs of *Trymatococcus amazonicus*; and 16, stems of *Ficus maxima*.

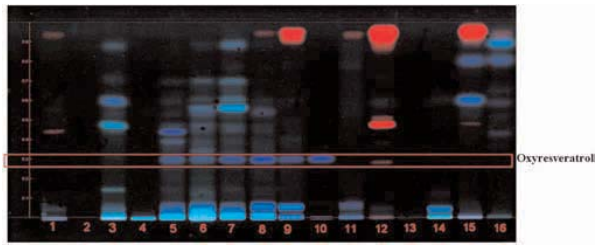

Figure 3

HPTLC comparison of *Morus species* with other related genera under UV 366 nm light. Tracks: 1, leaves of *M. alba* L.; 2, xylem of *M. alba* L.; 3, stems of *M. alba* L.; 4, fruits of *M. alba* L.; 5, roots of *M. alba* L.; 6, bark (BK) of *M. alba* L.; 7, bark of *M. alba* L.; 8, stems of *M. rubra* L.; 9, stems–leaves of *M. rubra* L.; 10, oxyresveratrol; 11, leaves of *M. rubra* L.; 12, leaves of *Maclura pomifera*; 13, fruits of *M. pomifera*; 14, roots of *M. pomifera*; 15, leaves–twigs of *Trymatococcus amazonicus*; and 16, stems of *Ficus maxima*.

which will be useful for identification of authentic samples (**Figures 2–4**).

4 Conclusion

This paper deals with the establishment of simple, rapid, specific, accurate, and precise HPTLC and HPLC methods for analysis of oxyresveratrol in *M. rubra* L. and *M. alba* L. Statistical analysis of the data showed the methods enable reproducible and selective analysis of oxyresveratrol. The detection wavelength for both methods was 327 nm. The mobile phase effectively resolved oxyresveratrol from the plant matrix and the methods can be used for both qualitative and quantitative analysis of oxyresveratrol in different plant samples. Moreover, chemical fingerprinting by both HPTLC and HPLC showed promise as methods for distinguishing between the various species and related genera.

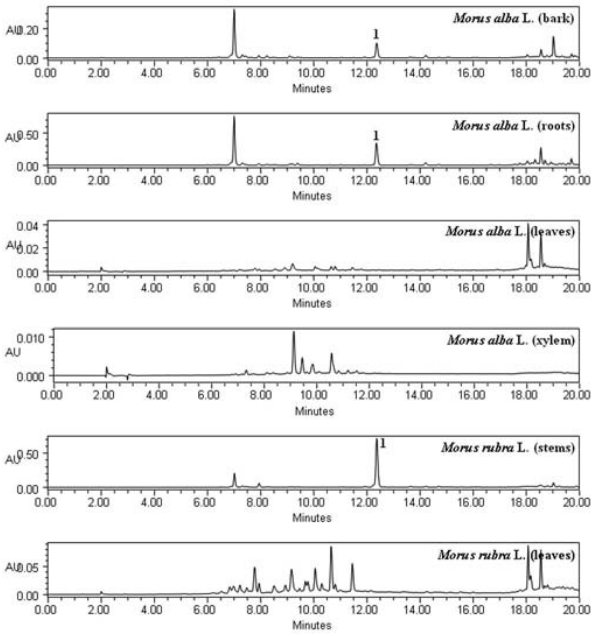

Figure 4

Typical HPLC chromatograms obtained from oxyresveratrol in different plant samples.

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